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▶ **The elution behavior of over 90 ninhydrin-reacting compounds, including some geometrical isomers, was studied with the automatic amino acid analyzer employing the conditions of Spackman, Moore, and Stein. Several compounds overlapped, often forming a sharp symmetrical peak. Some such peak mixtures were resolved with a change in the program temperature. Also identification of the components of a peak may be possible where sufficient differences in the 440- to 570-mμ absorption ratio of the ninhydrin reaction products occur. The study emphasizes the danger of a hasty identification of peaks found in the analysis of nonprotein fractions.**

METHODS of ion exchange chromatography for the resolution of mixtures of amino acids and related compounds have been developed by Moore and Stein (*9–11*). These procedures have since been further refined and adapted to an apparatus which automatically develops and records with quantitative precision the peaks eluted from the columns (*8, 12*).

During an investigation in this laboratory of the nonprotein nitrogen fraction of plant materials, a considerable number of peaks appeared which could not be identified with published chromatographic records (*8, 12*). In an effort to identify some of the peaks, a number of naturally occurring ninhydrin-reactive compounds, with elution volumes not already described, were chromatographed on the automatic analyzer. A considerable number of compounds had elution volumes close to, if not identical with, one or more other compounds. In some cases the 440- to 570-mμ color absorption ratios differed significantly to serve as a characteristic for identification. The behavior of some nonnitrogenous keto acids and lactones with ninhydrin on the automatic analyzer has been described previously (*17*).

A number of investigators have presented methods in ion exchange chromatography for resolving specific compounds which interfere with one another. However, the present study of the elution volumes of a large spectrum of naturally occurring nitrogenous compounds particularly found in plants was undertaken to provide some knowledge of their behavior under conditions widely employed in the automatic analysis of amino acids (*12*).

This work has further substantiated that misinterpretation of the identity of peaks found in nonprotein nitrogen fractions is wholly possible. Moreover, it confirms that peak symmetry need not be an index of the presence of a single component (*5*). An absolute identification must be based on independent criteria such as isolation of the compound responsible for the peak on scaled-up columns (*14*) followed by chemical and physical characterization, paper chromatography, and different ion exchange procedures.

This information should assist in the elucidation of unidentified peaks and point up specific instances where misidentification of others might result. Examples are pointed out where a relatively simple matter of changing the program temperature can partially or completely resolve a mixture. Changing the state of oxidation of sulfur compounds may also serve to displace the components. Quantitative evaluation of the peaks obtained with each program would provide additional evidence for the identity of the compounds.

EXPERIMENTAL

Methods. The separations described below were carried out on an amino acid analyzer, Model K-5000 (Phoenix Precision Instrument Co., Philadelphia, Pa.). The resin, length of columns, buffers, ninhydrin reagent, flow rates, and forerun were nominally the same as those prescribed by Spackman, Stein, and Moore (*12*). In place of phenol, 0.1 ml. of octanoic acid per liter of buffer was employed as a preservative (*6*).

The program timing was modified slightly. The buffer change (pH 3.25 to 4.25) on the 150-cm. column was made after 8 hours and 40 minutes with the 50° C. separations. The dual temperature separations carried out on this column used a 30° to 50° C. shift simultaneous with the buffer change (pH 3.25 to 4.25) after 11 hours. Separations carried out on the 50-cm. column with pH 4.26 buffer employed a shift of column temperature from 30° to 50° C. after 10 hours.

The elution volumes of the compounds were ascertained by running them individually and in small groups until no ambiguity resulted. The ninhydrin color constants given in our Table I are often only approximations. However, they may be used as a guide for comparison purposes with those provided in Table I of Spackman *et al.*

(*12*), as they are corrected to the same basis.

Standards. The positions of over 90 compounds including some geometrical isomers, which give a positive ninhydrin reaction, were studied. Of these, only levulinic acid does not contain nitrogen, and all but *S*-methylglutathione, methionine sulfone, *S*-methylcysteine sulfone, and α-acetylornithine have been found in natural materials. The more common nitrogen compounds studied were obtained from commercial sources and were of a high degree of purity. The γ-L-glutamyl-*S*-methyl-L-cysteine sulfoxide was obtained by oxidation of the reduced form of the isolated peptide and *S*-methyl-L-cysteine sulfone from the reduced form of the amino acid. Methylglutathione and methylmethionine sulfonium iodide were synthesized by methylation of the corresponding peptide and amino acid. A sample of the latter was also obtained from a commercial source. Isoasparagine (3-aminosuccinamic acid) was prepared in the laboratory. The remaining compounds were obtained from private individuals and are acknowledged.

RESULTS AND DISCUSSION

The 440- to 570-mμ Absorption Ratio. The ninhydrin reaction products of the largest number of compounds in this study have a 440- to 570-mμ absorption ratio in the range of 0.22 to 0.29. Some have somewhat higher ratios—i.e., the absorptions at both wavelengths are more nearly alike—and in a relatively few cases the ratios are unusually high (0.50 and up). The latter in combination with their elution volumes are of such a distinctive character as to aid in the identification of the compound; glutamic acid, 29 (number of compound in Table I and on figures), cystine, 40, and pipecolic acid, 43, are notable examples. Use of some of these higher absorption ratios for identification has been pointed out by Spackman *et al.* (*12*).

The five-membered imino acids and some nonnitrogenous compounds (*17*) have a greater absorption at 440 than 570 mμ. This generally provides easy recognition of the compound when considered with its elution volume.

While the absorption ratios of these two wavelengths will vary somewhat with the absorption cell and filter of each instrument, the ratio obtained employing given equipment and specified conditions was rather constant. Even in those cases where the 440- to 570-mμ absorption ratios show relatively small differences, it is often possible to ascertain the identity of a peak when a second or third compound is present as a contaminant in no more than traces. When large differences in ratios exist, peak identification will tolerate larger quantities of contaminating compounds. Conversely, in those instances where a peak ratio consistently falls between that of known compounds with the same elution volume, it is indicative of a mixture. The absorption ratios shown in Table I are given for some of the compounds which interfered with each other during the separations depicted in Figures 1a, 1b, 1c, and 2.

Elution volumes reported by various investigators presumably using almost identical equipment and conditions may vary. These variations may be the result of differences in column length and diameter, resin cross-linkage and particle size, and rather subtle differences in flow rates. Thus previously reported compounds were run as markers in this study. Elution volumes recently reported by Frimpter and Bass (*2*) are at considerable variance with some of those described here (Table I) presumably employing similar though not identical conditions. Figures 1a, 1b, and 1c are composites assembled from many separations. Figure 2 represents separations of actual mixtures containing the compounds listed.

150-cm. Column. Improved Separations with the 30° to 50° C. Program. For those components eluted from the column within the first 11 hours of the 30° to 50° C. program, this is in reality a 30° C.

Table I. Ratios Calculated from Absorption at 440 and 570 mμ of the Ninhydrin

No. of compound[a]	Compound	440/570, mμ	Color constant[b]	Other references[c]
1	Cysteic acid			(*12*)
2	DL-*o*-Phosphoserine			(*12*)
3	DL-*o*-Phosphothreonine			
4	γ-L-Glutamyl-*S*-methyl-L-cysteine sulfoxides		25.7	
5	γ-Glutamylmethionine sulfoxides			
6	Taurine			(*12*)
7	Levulinic acid	1.36	0.402 (440 mμ) 0.295 (570 mμ)	
8	*S*-Methyl-L-cysteine sulfone	0.229	24.1	
9	*S*-Methyl-L-cysteine sulfoxides	0.233	25.4	
10	*S*-Methylglutathione		21.2	
11	L-Hydroxyproline	5.95		(*12*)
12	γ-L-Glutamyl-*S*-methyl-L-cysteine		25.7	
13	DL-Methionine sulfoxides [2-amino-4-(methylsulfinyl)-butyric acids]			(*12*)
14	DL-Aspartic acid			(*12*)
15	Glutathione (reduced)	0.238	18.3	(*8*)
15A	Glutathione (oxidized)	0.236	36.6	(*8*)
16	DL-Methionine sulfone [2-amino-4-(methylsulfonyl)-butyric acid]			(*12*)
17	γ-L-Glutamyl-β-alanine	0.231	24.6	
18	γ-L-Glutamyl-β-aminoisobutyric acid	0.238	24.0	
19	L-Threonine	0.222		(*12*)
20	L-Serine			(*12*)
21	2-Azetidinecarboxylic acid	0.362	10.2	
22	L-Asparagine	0.247		(*12*)
23	δ-Acetylornithine (N^{δ}-acetylornithine)	0.236	25.3	
24	L-Glutamine			(*12*)
25	γ-Glutamylmethionine			
26	DL-Homoserine	0.246	24.2	(*4*)
27	DL-Sarcosine			(*12*)
28	*S*-Carboxyethyl-L-cysteine	0.269		
29	L-Glutamic acid	0.519		(*12*)
30	γ-Glutamylleucine	0.239	23.1	
31	L-Lanthionine (3,3′-thiodialanine)		24.6	
31A	*meso*-Lanthionine (3,3′-thiodialanine)		24.6	
32	L-Proline	5.98		(*12*)
33	DL-Citrulline	0.276		(*12*)
34	*S*-Methyl-L-cysteine	0.245	25.9	(*2*)
35	4-Methylproline	25.0		
36	Glycine	0.239		(*12*)
37	DL-α-Aminoadipic acid (2-aminohexanedioic acid)	0.283		(*12*)
38	Alanine			(*12*)
39	DL-α-Amino-*n*-butyric acid (2-amino-*n*-butyric acid)	0.236		(*12*)
40	L-Cystine	0.512		(*12*)
41	L-Homocitrulline	0.290	26.3	(*3, 13*)
42	L-Valine	0.231		(*12*)
43	DL-Pipecolic acid	0.947	1.21	
44	γ-L-Glutamyl-L-tyrosine			
45	γ-L-Glutamyl-L-phenylalanine		23.2 (50° C.) 27.1 (30° C.)	

system. Where this applies these terms are used interchangeably.

Citrulline, 33, and proline, 32, failed to separate at 50° C. but did so in the dual temperature program. In the former instance, obvious differences in absorption ratio would generally indicate the presence of a mixture. Glycine, 36, and α-aminoadipic acid, 37, yield a single symmetrical peak at 50° C. but are well separated at 30° C.

Homocitrulline, 41, is fully resolved from all compounds studied using the 30° to 50° C. system but is only partially separated from valine, 42, with the 50° C. program. Absorption ratio differences may prove to be of some use in identification of the two peaks.

The dual temperature provides a partial separation for leucine, 53, and leucaenol, 54, which have identical elution volumes at 50° C. Moreover, differences in the absorption ratios usually permit recognition of the peaks when they are partially resolved. *meta*-Carboxyphenylalanine, 56, also has shown improved separation from the leucine-leucaenol, 53–54, region with the 30° to 50° C. program.

IMPROVED SEPARATIONS WITH THE 50° C. PROGRAM. At 50° C., γ-glutamyl-*S*-methylcysteine, 12, showed much improved resolution from aspartic acid, 14, over that at 30° C. However, it was in the glutamic acid, 29, region of the chromatography that the 50° C. program afforded the much improved resolving power. *S*-Methyl-L-cysteine, 34, separated from glutamic acid, 29, whereas at 30° C. a single symmetrical peak resulted. In addition, γ-glutamylleucine, 30, is almost fully separated from both. γ-Glutamylmethionine, 25, was fully separated from the foregoing compounds and *S*-carboxyethyl-L-cysteine, 28, was resolved sufficiently from glutamic acid, 29, for quantitative estimation. At 30° C., however, γ-glutamylmethionine, 25, *S*-carboxyethyl-L-cysteine, 28, glutamic acid, 29, *S*-methyl-L-cysteine, 34, and a portion of γ-glutamylleucine, 30, are found in a single broadened peak. If one is concerned with an analysis for glutamic acid, 29, and citrulline, 33, the higher temperature provides sharper resolution. In all of the above cases glutamic acid, 29, can be distinguished by its uncommonly high 440- to 570-mμ absorption ratio. Depression of this ratio would suggest the presence of one or more compounds with a similar elution volume.

On the columns employed in this study, no resolution was obtained in the 30° to 50° C. system between α-amino-*n*-butyric, 39, and α-aminoadipic, 37, acids whereas at 50° C. excellent separation resulted, although the latter and glycine, 36, produced a single peak. The slightly higher ratio given by α-aminoadipic acid, 37 (compared with glycine, 36, and α-amino-*n*-butyric acid, 39) may be of assistance in identification, especially if one of the compounds predominates in either of the two mixtures.

In the 30° to 50° C. program, cystine, 40, and pipecolic acid, 43, are partially superimposed but full resolution is accomplished in the 50° C. system. An improved though still incomplete separation of homocystine, 61, and β-alanine, 62, occurs with the 50° C. program. A sufficient difference in ratio values should distinguish the identity of the two peaks.

FAILURE TO SEPARATE IN EITHER PROGRAM. In a number of cases two or more compounds fail to separate from each other in either of the two programs discussed. Cysteic acid, 1, phosphoserine, 2, and phosphothreonine, 3, form a single peak. Asparagine, 22, glutamine, 24, and δ-acetylornithine, 23, yield a perfectly symmetrical peak. In the 50° C. system, serine, 20, also has an elution volume identical with the preceding three compounds. Ratio differences were found to be useless in distinguishing one from the other and hence the presence of a mixture. Therefore, other means must be employed to ascertain the purity of the peak.

While hydroxyproline, 11, can be readily recognized by its high 440- to

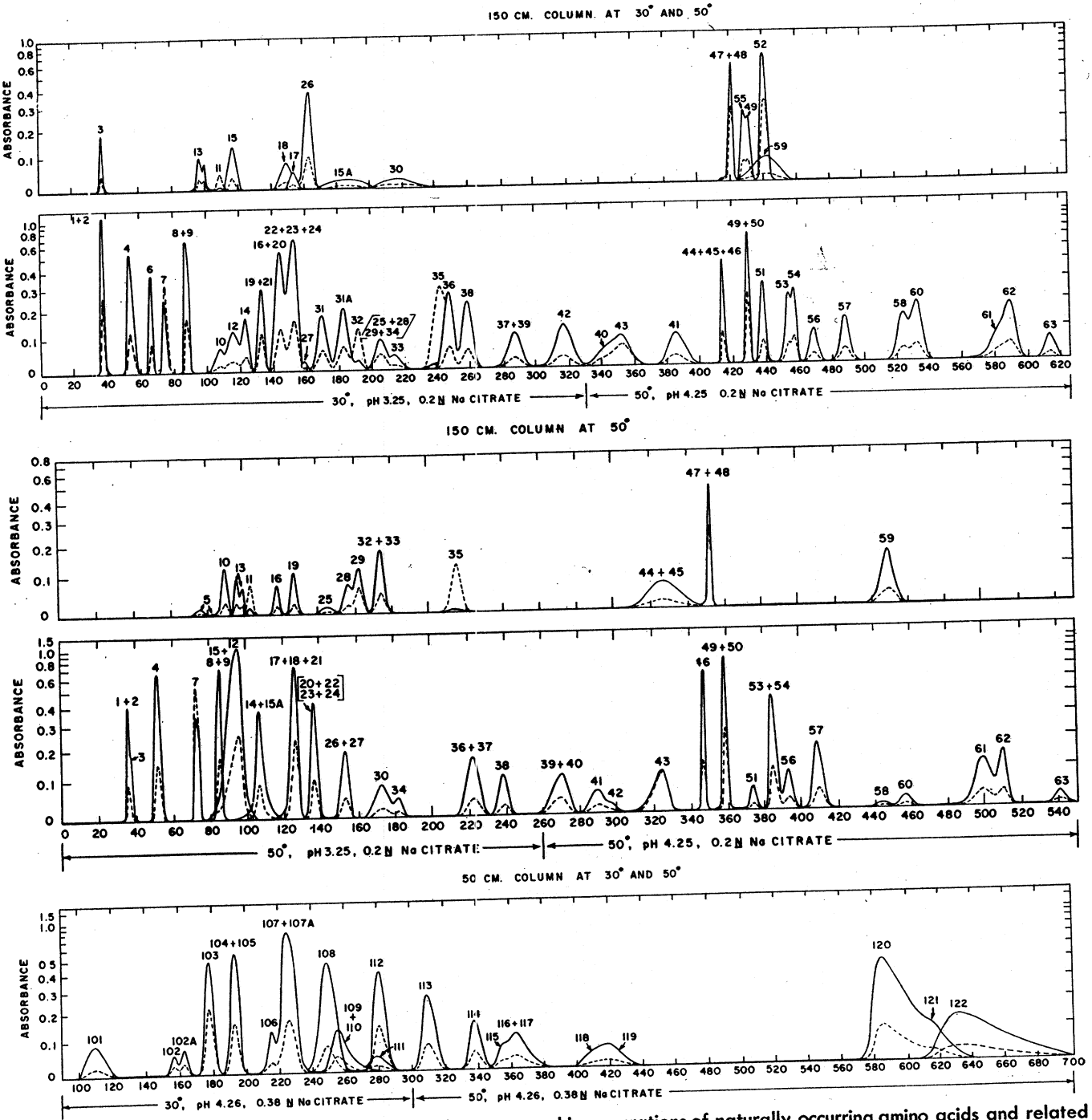
Reaction Product and Color Constants of Some Amino Acids and Related Compounds

No. of compound[a]	Compound	440/570, mμ	Color constant[b]	Other references[c]
46	*meta*-Carboxy-α-phenylglycine (α-amino-α-carboxy-*m*-toluic acid)			
47	DL + *meso*-Cystathionine	0.321		(*12*)
48	L-Djenkolic acid	0.524	46.3	(*2*)
49	DL-Methionine			(*12*)
50	L + *meso*-α,ε-Diaminopimelic acid (2,6-diaminoheptanedioic acid)		37.9	(*2*)
51	Isoleucine			(*12*)
52	Isoasparagine (3-Aminosuccinamic acid)			
53	L-Leucine	0.224		(*12*)
54	Leucaenol {β-[*N*-(3-hydroxypyridone-4)]-2-aminopropionic acid}	0.300	23.9	
55	α-Acetylornithine (N^2-acetylornithine)			
56	*meta*-Carboxyphenyl-L-alanine	0.263	29.4	
57	DL-3,4-Dihydroxyphenylalanine	0.265	24.6	
58	L-Tyrosine			(*12*)
59	D-Glucosamine	0.228		(*12*)
60	DL-Phenylalanine			(*12*)
61	DL-Homocystine	0.302		(*2, 4, 12*)
62	β-Alanine	0.260		(*12*)
63	β-Aminoisobutyric acid (2-methyl-β-alanine)			(*12*)
101	D-Galactosamine			(*12*)
102	5-DL-Hydroxylysine			(*12*)
102A	5-*allo*-Hydroxylysine			(*12*)
103	γ-Aminobutyric acid (4-amino-*n*-butyric acid)	0.490		(*12*)
104	L-Ornithine	0.373		(*12*)
105	L-2,4-Diaminobutyric acid	0.283		(*2*)
106	Ethanolamine	0.227		(*12*)
107	Ammonia	0.224		(*12*)
107A	L-Cysteinylglycine	0.204		
108	L-Methionine methyl sulfonium iodide [(3-amino-3-carboxypropyl) dimethyl-sulfonium iodide]	0.263	23.2	
109	L-Lysine	0.400		(*12*)
110	L-Kynurenine	0.236	23.2	
111	DL-5-Hydroxytryptophan	0.368		
112	DL-1-Methylhistidine	0.336		(*12*)
113	L-Histidine			(*12*)
114	L-3-Methylhistidine	0.313		(*12*)
115	L-Canavanine	0.415	25.7	(*15*)
116	L-Tryptophan	0.342		(*12*)
117	L-Anserine (β-alanyl-L-1-methylhistidine)			(*12*)
118	Creatinine			(*12*)
119	L-Carnosine (β-alanyl-L-histidine)			(*12*)
120	L-Arginine	0.248		(*12*)
121	DL-Homocysteine thiolactone	0.295	20.9	
122	L-3,5-Diiodotyrosine	0.262	24.4	

[a] Compounds in the 100 series were separated on the 50-cm. column.
[b] Approximate values which have been corrected to a basis comparable with those given by Spackman *et al.* (*12*).
[c] Reference to elution volume reported by others using systems similar to those employed in this study.

570-mμ absorption ratio, the compound is incompletely separated from aspartic acid, 14, at 50° C. and is mixed with the sulfur-containing dipeptide, 12, and tripeptide, 15, at 30° C. In studies on hydrolyzed protein and nonprotein fractions, cleavage of these peptides would render 30° C. more suitable for the determination of hydroxyproline, 11.

S-Methyl-L-cysteine sulfone, 8, and the two geometrical isomers of *S*-methyl-L-cysteine sulfoxide, 9, produce a single sharp peak with either program. In contrast, the geometrical isomers of methionine sulfoxide, 13, and methionine sulfone, 16, appear as three individual peaks. In view of the latter a careful study was made to ascertain whether the *S*-methyl-L-cysteine sulfoxides, 9, were being further oxidized on application to the column, or whether the thiodiglycol in the buffer might conceivably have reduced the sulfone, 8. The isomeric *S*-methyl-L-cysteine sulfoxides, 9, were prepared from *S*-methyl-L-cysteine, 34, (1.0 mmole) by overnight exposure to hydrogen peroxide (1.1 mmoles) in the refrigerator. The reaction mixture was overloaded on the 150-cm. analytical column and chromatographed at 30° C. with pH 3.25, 0.2*N* sodium citrate buffer. The eluate containing the heavy band of material



Figures 1a, 1b, and 1c. Composites of several chromatographic separations of naturally occurring amino acids and related compounds on columns of Amberlite IR-120

------- 440 mμ, ——— 570 mμ

For clarity, the compounds are shown in Figures 1a and 1b as two curves based on the same effluent volumes. The numbers associated with the various peaks refer to the compounds listed in Table I

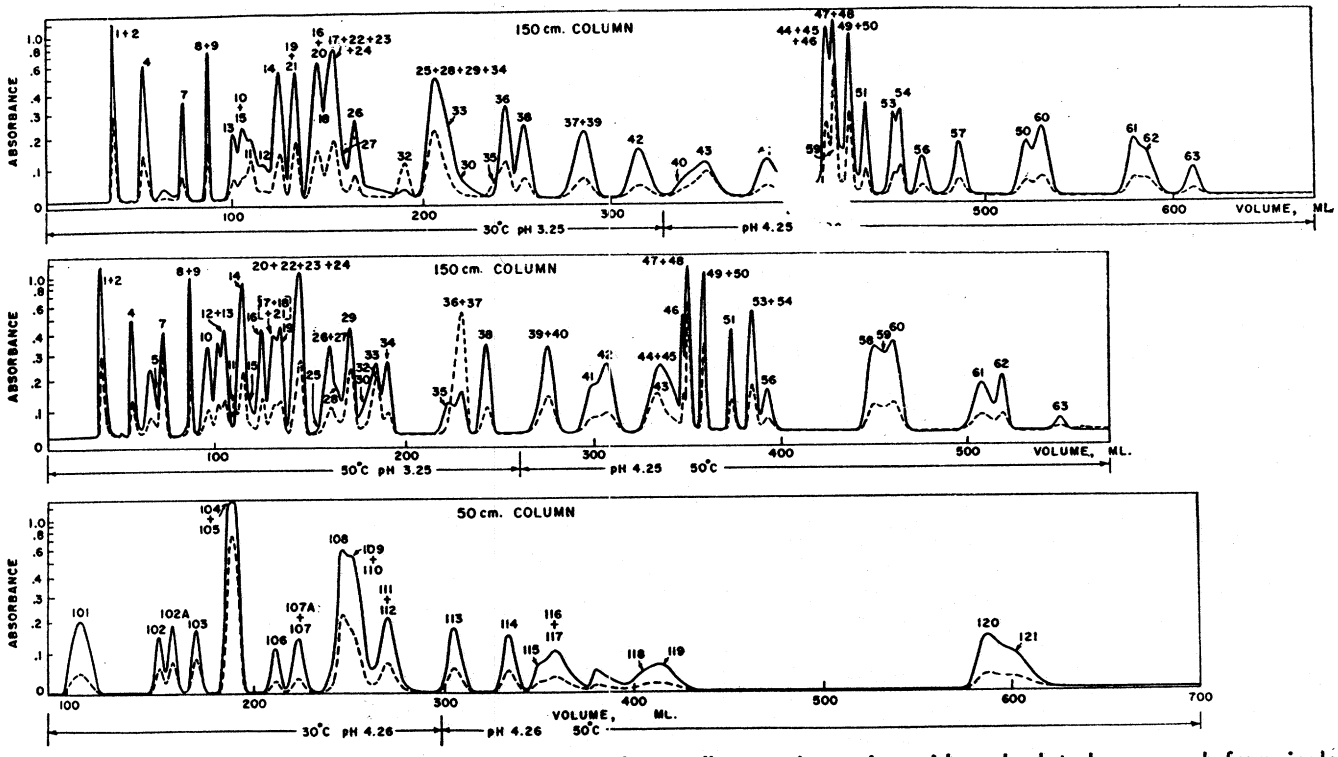


Figure 2. Tracings of the chromatographic separations of naturally occurring amino acids and related compounds from single mixtures in single runs on columns of Amberlite IR-120

------- 440 mμ, ——— 570 mμ

The numbers associated with the various peaks refer to the compounds listed in Table I. In the middle curve, No. 12 is mixed with the slower moving isomer of No. 13

was collected through the manifold of the analyzer before mixing with the ninhydrin reagent could occur. The eluate was desalted on Dowex-2 (*1*) and studied using one dimensional paper chromatography with phenol to water (80 to 30). In a similar overloading of the analytical column, *S*-methyl-L-cysteine sulfone, 8, (0.11 mmole) was chromatographed, recovered unreacted, and desalted for examination on paper chromatograms. The recovered sulfone, 8, gave a single yellowish brown ninhydrin spot, R_F = 0.46, identical with the standard which had not been put through the ion exchange column. The recovered *S*-methyl-L-cysteine sulfoxides, 9, produced a single gray-violet ninhydrin-positive spot with an R_F = 0.62 in agreement with the natural *S*(+)-methyl-L-cysteine sulfoxide, 9, isomer while no evidence was obtained for the presence of the sulfone, 8. Paper chromatography employing methanol to water to pyridine (80 to 20 to 4) (*16*) resolved the two geometrical sulfoxide isomers. Rechromatography on the IR-120 column of a mixture of the recovered sulfone and isomeric sulfoxides yielded a single sharp peak. The absorption ratios are almost identical for both compounds.

At 50° C., reduced glutathione, 15, overlaps the *S*-methyl-L-cysteine sulfoxides, 9, and sulfone, 8, peak while the oxidized form (*S-S*) appears mixed with aspartic acid, 14, as a broad zone. At 30° C., reduced glutathione, 15, is eluted with γ-L-glutamyl-*S*-methyl-L-cysteine, 12, and oxidized glutathione, 15A, (*S-S*) occurs as a spread peak in front of, and partially superimposed with proline, 32.

In this investigation a fresh supply of reduced glutathione, 15, produced a single peak of the sulfhydryl compound on column chromatography. However, in practice it is most difficult to avoid some air oxidation of tissue extracts converting part to the disulfide form. It would therefore simplify matters if all the glutathione were converted to the disulfide form by air oxidation before chromatography.

Treatment of reduced glutathione with hydrogen peroxide oxidized part of it to the disulfide form and also produced a second compound which gave a peak overlapping that of cysteic acid, 1, and the phosphohydroxyamino acids, 2 and 3. This second compound produces a peak probably identical in position with that from glutathione-*S*-sulfonate described by Moore *et al.* (*8*). In those cases where sulfur compounds overlap with other components, it has often been possible by changing the state of oxidation to reorient the compound to a position which permits both identification and quantitative determination.

2-Azetidinecarboxylic acid, 21, has an elution volume identical with threonine, 19, at 30° C., and at 50° C. is mixed with threonine as well as γ-L-glutamyl-β-alanine, 17, γ-L-glutamyl-β-aminoisobutyric acid, 18, and methionine sulfone, 16. Although 2-azetidinecarboxylic acid, 21, has an absorption ratio unlike its 5- and 6-membered homologous imino acids, it is sufficiently higher to be distinguished from the four other compounds eluted in this region; the four, however, cannot be recognized from each other by such a method.

γ-L-Glutamyl-L-tyrosine, 44, and γ-L-glutamyl-L-phenylalanine, 45, failed to resolve from each other in both programs. In the 30° to 50° C. system, the picture is further complicated by the presence of *meta*-carboxy-α-phenylglycine, 46, in this region, while in the 50° C. program, pipecolic acid interferes. Nevertheless, the presence of pipecolic acid, 43, is normally readily discernible by its 440- to 570-mμ absorption ratio of nearly unity.

α-ε-Diaminopimelic acid, 50, and methionine, 49, form a single peak and although cystathionine, 47, and djenkolic acid, 48, form another, the considerably higher absorption ratio of the latter compound provides a method of discrimination.

Glucosamine, 59, at 50° C., has an elution volume conflicting with tyrosine, 58, and phenylalanine, 60, and in the 30° to 50° C. system appears as a broad peak blanketing the methionine-

isoleucine-leucine region, 49-51-53. Spackmen *et al.* (*12*) have found glucosamine, 59, immediately following leucine, 53, at 30° to 50° C. Hamilton and Anderson (*5*) and Moore (*7*) have pointed out that the position of the glucosamine peak is relatively unaffected by the pH of the eluting buffer. Thus, the position of the tyrosine, 58, and phenylalanine, 60, peaks may be altered with respect to the amino sugar, 59, by an appropriate change in the time of the buffer shift.

50-cm. Column. On the 50-cm. basic column ornithine, 104, and 2,4-diaminobutyric acid, 105, form a perfectly symmetrical peak but can be distinguished by their somewhat different 440- to 570-mμ absorption ratios; ornithine, 104, has a higher ratio. Lysine, 109, and kynurenine, 110, have similar elution volumes but the latter tends to tail. Methionine methyl sulfonium salts, 108, also overlap the lysine-kynurenine peak, 109-110; however, lysine has a notably higher absorption ratio than the other two compounds.

The peaks of 5-hydroxytryptophan, 111, and 1-methylhistidine, 112, are superimposed and might well prove difficult to distinguish by ratio. This also applies to the single ammonia-cysteinylglycine peak, 107-107A (of which the latter is probably cystinylglycine as a result of ready oxidation).

Canavanine, 115, appears as a shoulder on the tryptophan-anserine, 116-117, peak which in this study failed to separate from each other. Canavanine, 115, has the highest ratio of the three and this might be of assistance in identification. Homocysteine thiolactone, 121, was found as a shoulder on the trailing side of arginine, 120, and might be overlooked, particularly if the latter were present in considerably larger amounts than the former. Ratio differences are too small to be a dependable means of identifying one in the presence of the other. Of all the compounds examined, 3,5-diiodotyrosine, 122, exhibited the greatest retention on this column and was for the most part eluted just beyond the arginine-homocysteine thiolactone, 120-121, region.

2,3-Diaminopropionic acid (not shown in the figures) was found to be eluted on the 50-cm. column at a position just beyond and partially overlapping the ammonia peak. This is in agreement with the unpublished data of Van Etten of the Northern Utilization Research and Development Division, Peoria, Ill.

ACKNOWLEDGMENT

The authors thank John F. Thompson and C. J. Morris, U. S. Plant, Soil and Nutrition Laboratory, Ithaca, N. Y., for samples of α- and δ-acetylornithine, *meta*-carboxyphenylalanine, *meta*-carboxy-α-phenylglycine and the γ-glutamyl dipeptides. Thanks are also due L. Fowden, University College, London, for a sample of 2-azetidinecarboxylic acid, and to L. Weil and T. J. Fitzpatrick of this laboratory for the *S*-carboxyethylcysteine, and isoasparagine, respectively.

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RECEIVED for review June 12, 1962. Accepted September 13, 1962. Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Reference to a company or product does not imply approval or recommendation by the U. S. Department of Agriculture to the exclusion of others that may be suitable.